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Original Article

Molecular weight and ionic strength dependence of fluorescence intensity of the Calcofluor/β-glucan complex in flow-injection analysis ☆

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Abstract

The flow-injection analysis (FIA) method has long been used for the quantitative determination of β-glucans. FIA makes use of the enhanced fluorescence produced when β-glucan forms complexes with Calcofluor. For successful application of this analysis method, the lowest molecular weight (MW) in the sample has to be higher than a critical MW below which the fluorescence intensity of the complex becomes weaker.

Fluorescence measurements reveal that the fluorescence intensity of the Calcofluor/β-glucan complex is strongly dependent on the ionic strength of the solution. At high ionic strength, fluorescence intensity is high and critical MW is low. At low ionic strength, on the other hand, fluorescence intensity is low and critical MW is high. Turbidity measurement shows that Calcofluor/β-glucan complex forms aggregates in the solution and the rate of aggregate formation depends on the MW of β-glucan and concentration of Calcofluor. This study indicates that the FIA method has to be used with solutions of high ionic strength and a high Calcofluor to β-glucan ratio to broaden the applicable MW range of β-glucan analysis. Application of FIA method to the analysis of β-glucan in oat products turns out to be successful as confirmed by comparison with conventional enzymatic analysis.
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Keywords: β-Glucan; Flow-injection analysis; Calcofluor; Ionic strength

1. Introduction

Oat products have long been associated with health and more recently with its hypochole-sterolemic property first reported by De Groot et al. (1963). Additional research by Anderson and Wood (1990) and others including Schneeman and Gallaher (1985) and van Horn et al. (1988) have established its usefulness in lowering blood cholestrol. The oat products reported to have these properties are the conventional oat flakes, flour, and bran along with some other processed products including Oatrim and Nutrim (Inglett and Carriere, 2001). A principal component for oat biological activity appears to be its β-glucan component.

β-Glucan is a water-soluble polysaccharide present in cereal brans and became a commercial product because of its effectiveness in reducing serum cholesterol. This natural component of dietary fiber is a nondigestible polysaccharide. When developing healthier foods containing β-glucan, it is important that its content be determined with an accurate method. β-Glucans are linear polysaccharides composed of β-D-glucopyranosyl units linked (1→3). A few chain segments have as many as 4–8 consecutive units with (1→4)-linked sugar units. About 70% of the chain links are the (1→4) type (Whistler and BeMiller, 1999).

There are two widely used methods for β-glucan determination. One is based on the utilization of specific enzymes and the determination of released oligosaccharide or glucose (McCleary and Glennie-Holmes, 1985). The other is based on the specific binding of Calcofluor to β-glucan (Wood, 1980a, b, 1982). This binding results in an increase in fluorescence intensity that is proportional to the concentration of β-glucan in solution. From these findings, a method to quantify β-glucan called

☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Calcofluor-flow-injection analysis (FIA) was developed and was applied for the analysis of β-glucans in beer and wort (Jörgensen et al., 1985; Jörgensen and Aastrup, 1986; Mekis et al., 1987; Sendra et al., 1989).

There are two major restrictions in employing the FIA method for quantitative determination of β-glucan: applicable low molecular weight (MW) limit and ionic strength dependence. The FIA method does not work with low MW glucans. This low MW limit had been reported around 9200 (Gomez et al., 2000). This experimental observation suggests that there should be MW dependence of fluorescence intensity around the MW detection limit or in the entire MW range. Since the mechanism by which the Calcofluor/β-glucan complex develops enhanced fluorescence is not known, this hypothesis can only be examined by actual experimentation.

For the evaluation of MW distribution in the sample, β-glucan fractions isolated by size-exclusion chromatography (SEC), both off-line (Foldager and Jörgensen, 1984; Manzanares et al., 1991) and on-line post-column detection (Wood et al., 1991; Gomez et al., 1997; Knuckles et al., 1997) had been employed. Since refractive index (RI) detector does not discriminate target material from unwanted impurities like starches, fluorescence intensity measurement from the Calcofluor/β-glucan complex is the most commonly used technique; however, this technique assumes that the fluorescence intensity from the Calcofluor/β-glucan complex is not dependent on MW of β-glucan. If this assumption does not hold, the relative distribution of each MW component has to be taken into consideration. Even if we are interested in the total amount of β-glucan in the sample, MW-dependent fluorescence intensity will lead us to overestimation or underestimation of the measured quantity of β-glucan depending on the distribution of MW in the sample.

Recently, MW standards of β-glucan became commercially available. Therefore, we can investigate the MW dependence without time-consuming process of isolation and MW determination. In this report, we investigated the MW effect on the fluorescence intensity of the Calcofluor/β-glucan complex at several ionic strengths. The turbidity measurement helps understanding the MW effect seen in the formation of the complex. From these experimental results, optimum conditions for the FIA method will be discussed.

2. Materials and methods

2.1. Chemicals

β-Glucan MW standards were purchased from Megazyme (Wicklow, IR) and the MWs were 40,000, 123,000, 183,000, and 359,000 g/mol. The MW of each MW standard was confirmed with a Gel Permeation Chromatography (Shimadzu, VP series, Tokyo, Japan) equipped with a light scattering detector (Dawn EOS) and an RI detector (Optilab DSP) from Wyatt Technology (Santa Barbara, CA, USA).

Calcofluor (Fluorescent Brightener 28), sodium phosphates (dibasic and tribasic), and sodium hydroxide were all purchased from Aldrich (Milwaukee, WI, USA). The water used was distilled water passed through a Milli-Q purification system (Millipore, Bedford, MA, USA). All the other chemicals were of analytical-reagent grade.

2.2. Equipment

For the fluorescence spectrum and determination of fluorescence intensity of the Calcofluor/β-glucan complex, a fluorometer from Perkin-Elmer (Model LS-50B, Wellesley, MA, USA) was utilized. For the transmittance measurement, a custom-built turbidometer was used. This instrument consists of a He–Ne laser, laser power meter, sample cell, and a computer interfaced with laser power meter. All operations were performed using custom-programmed software. Microscopic image of aggregates from Calcofluor/β-glucan complex was taken with a phase-contrast microscope (Diastar, Reichert, Deerfield, IL, USA) with the objective set at $10\times$. A Nikon digital camera (D100, Tokyo, Japan) was mounted at the top of the microscope via a microscope adapter (Universal Microscopic Adapter, Edmund Industrial Optics, Barrington, NJ).

2.3. Sample preparation

For the measurement of the fluorescence spectrum, 2×10^{-3}% Calcofluor solutions were prepared in phosphate buffers (3.125, 6.25, 12.5, 25, 50, and 100 mM, pH 10) to produce six different ionic strengths. β-Glucan solutions (2×10^{-2}%) were prepared in the same buffer for each MW standard. The Calcofluor solutions were then titrated with β-glucan solutions to monitor the variation in the spectrum intensity. In order to accommodate the signal intensity of these solutions to the sensitivity of the fluorometer, a neutral density filter (OD = 1.5) was used on the detector side of optics when it is necessary.

For the turbidity and time-dependent fluorescence measurement, 0.4% β-glucan solutions were prepared in 50 mM phosphate buffer (pH 10) for each MW standard. A 0.4% Calcofluor solution was prepared as a stock solution in the same buffer. All MW standards were prepared by adding 40 mg β-glucan to 9.96 g phosphate buffer while stirring. The sample was then microwaved to boil and allowed to cool. Microwave heating was adopted for easy dissolution of β-glucan and to minimize loss of the solvent by shortening heating time. Buffer solution was added back to make up for evaporation. Standards were filtered through a 0.45 μm Durapore prior to mixing with the dye. The Calcofluor solution was diluted to a desired concentration and mixed with the β-glucan solution at a 1:1 ratio just prior to each measurement.

2.4. Simulated FIA set-up

In the typical FIA set-up, a constant flow of Calcofluor solution is delivered to the mixing coil by a peristaltic pump and a fixed volume of the sample solution is injected into the same column. They are mixed in a mixing coil and passes through a fluorescence detector. In our simulated FIA set-up, Calcofluor solution and the sample solution are premixed in a fluorometer cuvette and emission spectrum is taken in a fluorometer. Although the two set-ups use different types of cuvettes (flow cuvette vs. conventional cuvette), there is no difference in the signal detection scheme.

3. Results and discussion

The fluorescence spectrum of the Calcofluor/β-glucan complex is shown in Fig. 1. The excitations are at 260 nm and between 360 and 380 nm with emission spans from 370 to 600 nm range. For the following experiments, 360 nm was chosen as the excitation wavelength. The total integrated fluorescence intensity between 370 and 600 nm was defined as the fluorescence intensity from each sample solution. A neutral density filter (OD = 1.5) was used on the detector side of the fluorometer to accommodate the high intensity signal and to simulate the experimental situation for the FIA method.

Fluorescence intensities were examined as a function of the β-glucan concentrations. This procedure was a simulation for establishing a calibration curve for the FIA method. Two ionic strengths of buffer solutions, 3.125 and 100 mM buffer, were chosen for this study. A quantity of 40 μg of Calcofluor in a 2 mL buffer solution was titrated with 2×10^{-3}% β-glucan solutions. In the case of Calcofluor solution in 100 mM phosphate buffer, β-glucan with MW of 123,000, 183,000, and 359,000 g/mol showed superposition of fluorescence intensities, while MW 40,000 g/mol sample showed a little lower signal intensity (Fig. 2 (top)). The same type of experiment was performed with the lower ionic strength, 3.125 mM phosphate buffer solution (Fig. 2 (bottom)). In this case, the fluorescence intensity was lower than in the previous case. Titration results revealed an intensive MW dependence when the concentration of β-glucan was higher than that of Calcofluor. While the fluorescence intensities from high MW samples 123,000, 183,000, and 359,000 g/mol were superimposed at the beginning of titration, the signal from 40,000 g/mol sample was far weaker than those from higher MW samples. From the above two experiments, two conclusions were obtained for the quantitative determination of β-glucan with FIA method. First, the concentration of Calcofluor has to be the same or higher than that of the β-glucan in the FIA method. Both graphs in Fig. 2 show that the calibration curve bends when β-glucan/Calcofluor ratio was higher than unity. This bent curvature will result in large errors for high concentrations of β-glucan. MW-dependent fluorescence intensity, which appears in this region when low ionic strength buffer was used, makes the situation even worse. A slight bending of the calibration curve at the first half of the titration was caused by a dilution effect.

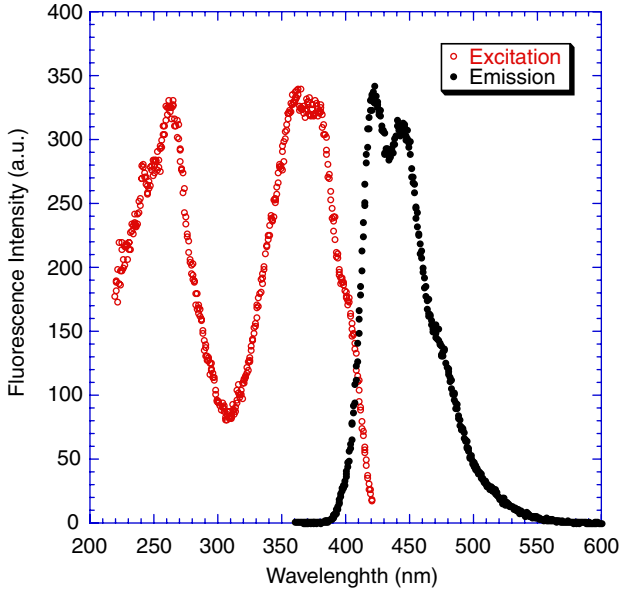

Fig. 1. Excitation and emission spectra of Calcofluor/β-glucan complex. Excitation was observed at 430 nm and emission was monitored with excitation wavelength of 360 nm.

Secondly, considering the low fluorescence-enhancing effect and insensitivity with low MW sample on the fluorescence intensity, solvents with low ionic strength should be avoided with the FIA method. Another advantage of using high ionic strength buffer is that the background signal intensity, i.e., the fluorescence intensity from Calcofluor without β-glucan, was ca. 10% lower and the signal-to-noise ratio was improved. As shown in Fig. 2, it is clear that the data from MW 40,000 are much closer to the other MW samples in higher ionic strength solution. This means the applicable MW limit is very close to 40,000 with an ionic strength of 100 mM but much higher than 40,000 but below 123,000 in 3.125 mM. Therefore, it is concluded that high ionic strength buffer broadens the applicable MW range in the FIA method. However, 40,000 is still too low MW for the application of the FIA method even with 100 mM buffer. One can speculate that applicable MW range can be more broadened by increasing the ionic strength of buffer. This issue is discussed below.

The above experimental results clearly show that both ionic strength and MW of β-glucan are important factors in the quantitative measurement of β-glucan. In the following experiment, ionic strength dependence and MW dependence were investigated at six buffer concentrations, 3.125, 6.25, 12.5, 25, 50, and 100 mM, and with two MWs, 40,000 and 359,000 g/mol, respectively. Concentrations of Calcofluor and β-glucan were chosen to prepare the same solution as the ones shown in Fig. 2 where 200 μL of substrate was added. In other words, 40 μg of Calcofluor in a 2 mL solution was mixed with 40 μg of β-glucan in a

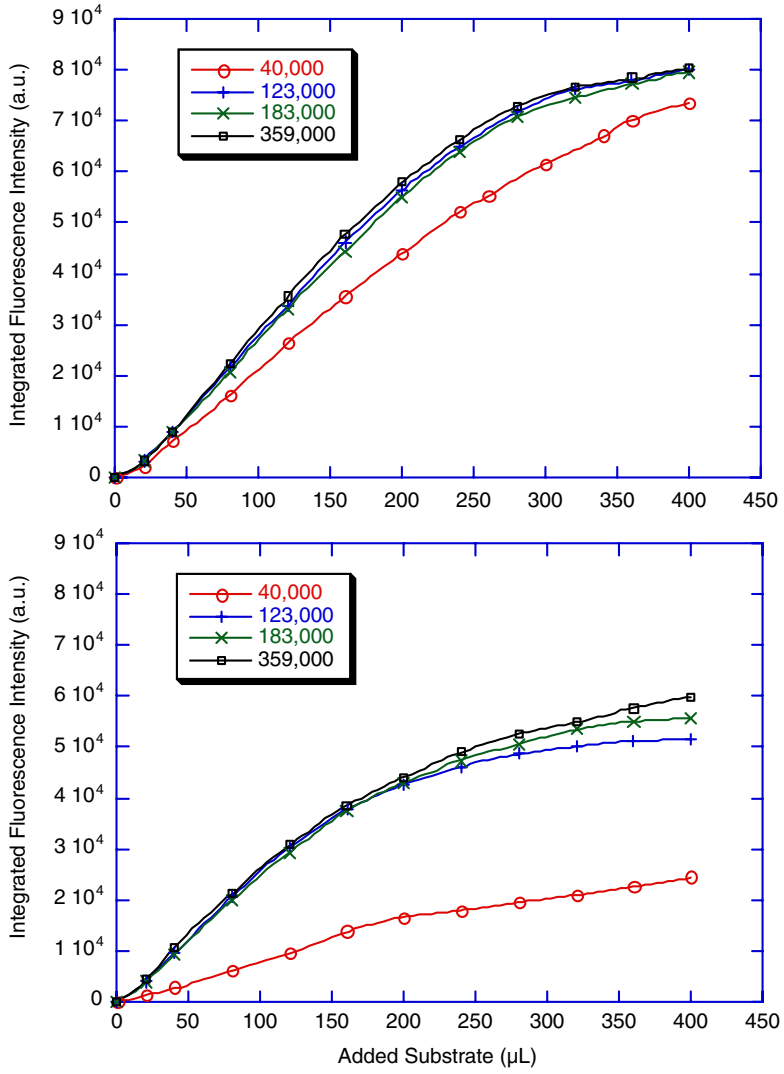

Fig. 2. Fluorescence intensity of Calcofluor/β-glucan complex in different ionic strength medium. (Top) In high ionic strength (100 mM) medium, 2 mL of Calcofluor solution (20 mg/L) was titrated with β-glucan solution (200 mg/L). (Bottom) In low ionic strength (3.125 mM) medium, 2 mL of Calcofluor solution (20 mg/L) was titrated with β-glucan solution (2 g/L).

200 μL solution. Since the two other MW samples, 123,000 and 183,000 g/mol, showed the same response as that of 359,000 g/mol sample at the given experimental condition (Fig. 2), these were not included in this experiment. As shown in Fig. 3, the experimental results showed that the fluorescence intensity from both high and low MW β-glucans increased as ionic strength of buffer increased. However, while the fluorescence intensities from high MW reaches plateau at high ionic strength, the intensity of the low MW continued to increase in the ionic strength range investigated. Although we are not sure if the two curves will meet at extremely high ionic strength medium, it is clear that the lower applicable MW limit of β-glucan would be lowered by using high ionic strength solvent. The two curves may meet at higher than 1000 mM buffer. However, this buffer concentration was too high to be practically adopted for the experimental condition. Hence, it is more reasonable to conclude that 40,000 is too low an MW for quantitative determination of β-glucans with the FIA method.

In principle, if Calcofluor does not interact with the SEC column material, it should be possible to use SEC columns for the fractionation of the Calcofluor/β-glucan complex. This trial failed because the peaks for the Calcofluor/β-glucan complexes appeared at an extremely high MW range regardless of their actual MW except for the lowest MW (40,000 g/mol). The peak from 40,000 g/mol sample also appeared at much higher than expected MW region, and the reproducibility of the experiment was poor. This means that Calcofluor/β-glucan complexes rapidly form large aggregates and the formation of the aggregates appeared MW dependent. In order to support this point of view, turbidity measurements were performed.

The precipitation (i.e., aggregate formation) of the Calcofluor/β-glucan complex was investigated by varying the Calcofluor/β-glucan weight ratio (Fig. 4) using 0.4%

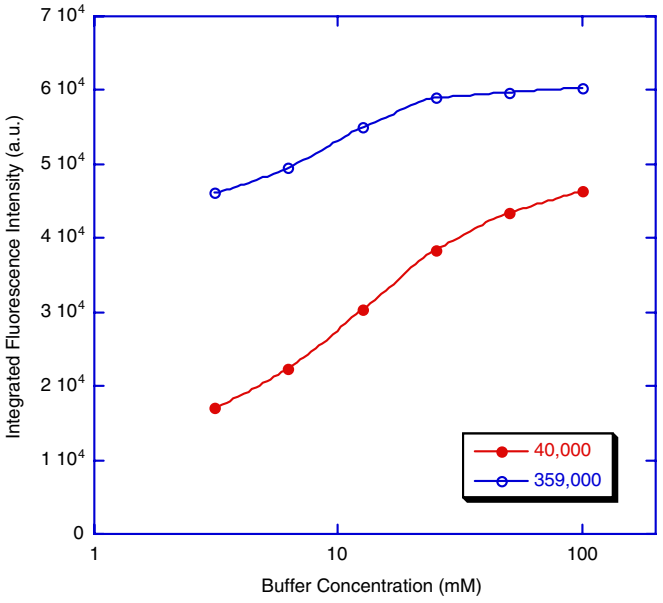

Fig. 3. Fluorescence intensity of Calcofluor/β-glucan complex as a function of ionic strength of phosphate buffer. Data from two molecular weights (40,000 and 359,000) are shown. Calcofluor/β-glucan weight ratio was fixed to 1:1.

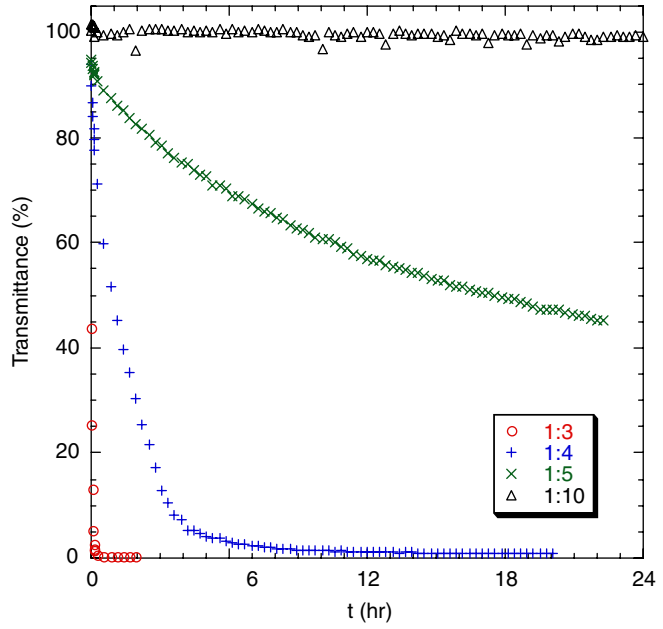

Fig. 4. Time dependence of aggregate formation of Calcofluor/β-glucan complex as monitored by turbidity measurement. Turbidity variation is shown with Calcofluor/β-glucan weight ratio as another variable. The molecular weight of β-glucan is 40,000.

40,000 g/mol β-glucan solutions mixed with various dilutions of 0.4% Calcofluor solution in a 50 mM phosphate buffer (pH 10). As the Calcofluor/β-glucan ratio was increased from 1/10 to 1/3, faster aggregate formation was induced. For the experimental data shown in Fig. 4, 10 times higher concentrations for β-glucan were chosen to show the overview of the trend of aggregate formation. According to our conclusion that was obtained from the data shown in Fig. 2 (top), the concentration of Calcofluor needs to be the same or higher than that of β-glucan to be applied in the FIA method. Fig. 4 suggests that the concentration of Calcofluor/β-glucan complex is another major factor that governs the formation of aggregates. When the SEC experiment is performed, the local concentration of Calcofluor/β-glucan complex will be increased as the sample solution passes through the column. It should be the reason why we observe the peaks at much higher than the expected MW range in the SEC data. Aggregate formation of Calcofluor/β-glucan complex may cause the same problem in obtaining fluorescence spectrum with the FIA method. If the fluorescence intensity is significantly decreased as Calcofluor/β-glucan complex form aggregates in the solution, it can be a source of experimental error since the rate of formation of aggregates depends on both concentration and MW of β-glucan. To evaluate this possibility, the time dependence of transmittance was monitored at the same experimental condition as that of Fig. 3 with 100 mM phosphate buffer. During 1 h experimental period, all four samples examined showed time-dependent aggregate formation (Fig. 5). Higher MW samples showed faster aggregate formation as was expected. If the time difference of Calcofluor/β-glucan complex from the moment of formation to the moment of detection was only a few seconds, aggregate formation should not be an interference factor. In the case of simulation experiment shown in Fig. 2, the duration of experiment per chosen MW sample, i.e., total titration

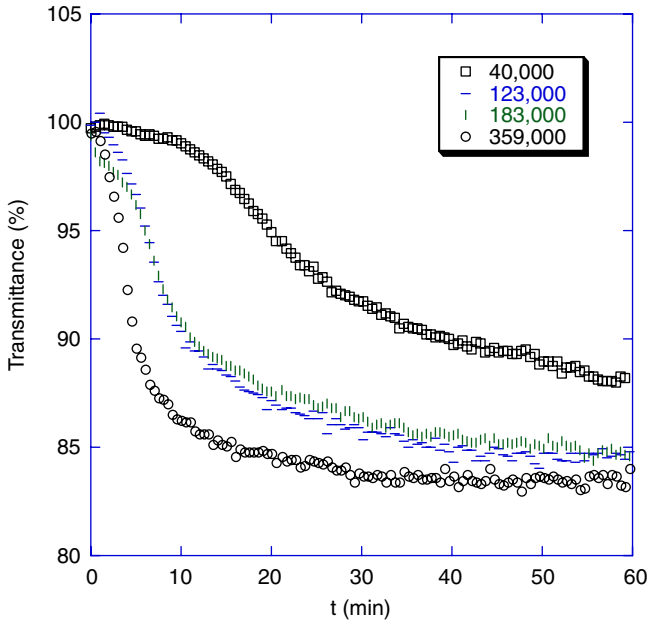

Fig. 5. Time dependence of aggregate formation of Calcofluor/β-glucan complex as monitored by turbidity measurement. Ionic strength of buffer solution was 100 mM and the concentration of β-glucan was 20 mg/L. It is shown that aggregates were formed more quickly with β-glucan with higher molecular weight.

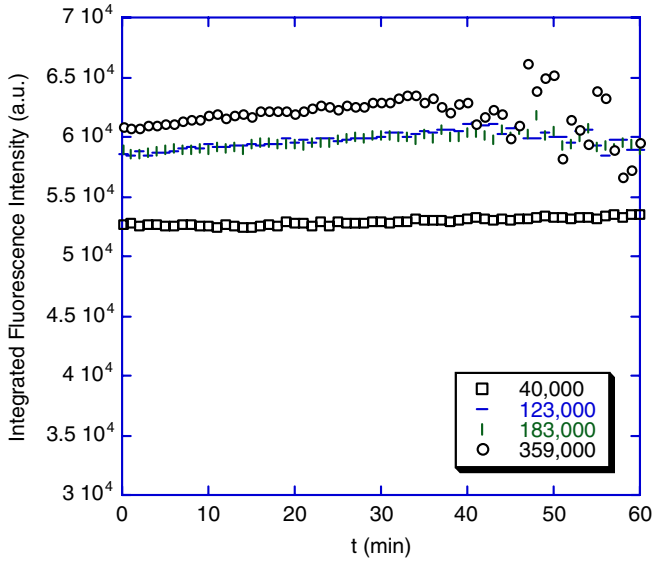

Fig. 6. Time dependence of integrated fluorescence intensity from Calcofluor/β-glucan (1:1 by weight) complex. Experimental condition is the same as above (Fig. 6). Fluctuation in the data from the highest molecular weight (359,000) is due to the formation of large aggregates.

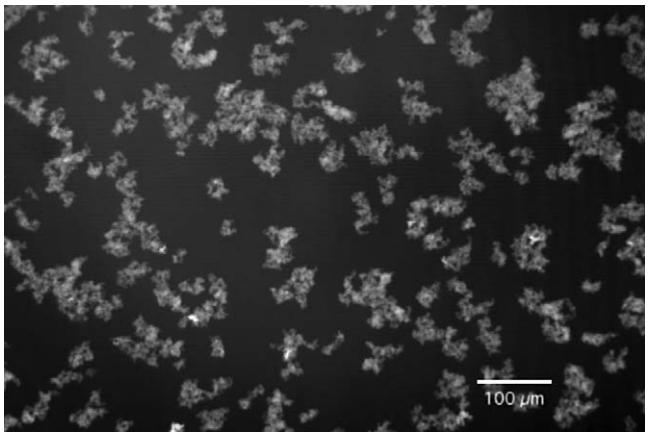

Fig. 7. Micrograph of aggregates of Calcofluor/β-glucan complex. This picture was taken from MW = 183,000 sample after the transmittance experiment shown in Fig. 5.

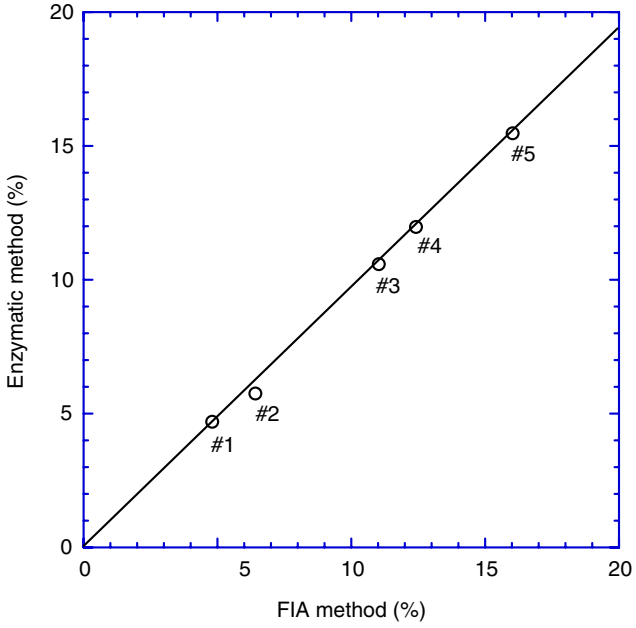

Fig. 8. Relationship between enzymatic method and FIA method for analysis of β-glucan in oat products, Oatrim and Nutrim (sample #1: commercial Nutrim produced by Van Drunen; sample #2: commercial Oatrim produced by Quaker Oats; sample #3: Oatrim prepared in the lab; sample #4: commercial Oat bran produced by Quaker Oats; sample #5: Nutrim prepared in the lab).

time, was about 30 min. Since the high MW β-glucans form aggregates very quickly (Fig. 5), the possible variation of the fluorescence intensity was monitored at the same experimental condition. The result was shown in Fig. 6. During the first 30 min of the experiment time, three low MW samples except the highest MW β-glucan showed very slight increase (less than 2%) in the fluorescence intensity. Considering the dramatic changes in transmittance shown in Fig. 5, this minor variation in fluorescence intensity is an unexpected result. The complex with MW 359,000 sample showed fluctuation in the output after 30 min. This result was confirmed by checking its reproducibility. Visual inspection of this sample revealed that large aggregates were the cause of this fluctuation. Eventually, the formation of aggregates led to a low signal intensity because of the precipitation. By comparing the two data in Figs. 5 and 6, it was shown that the fluorescence intensity was not affected much by the formation of aggregates unless they grow to be large enough for precipitation. It was again confirmed that the complex formed from high MW β-glucan grows faster. This experimental result suggests that the time gap between mixing of β-glucan with Calcofluor solution and its fluorescence detection has to be minimized especially when the MW of β-glucan is very high.

The morphology of the aggregates formed from Calcofluor/β-glucan complex is not dependent on the MW of β-glucan. A typical image of aggregates formed from MW 183,000 is shown in Fig. 7. The large size of the aggregates explains why SEC columns cannot be used with Calcofluor/β-glucan complex.

Knowing the required experimental condition for the analysis of β-glucan, Oatrim and Nutrim samples prepared in the lab and commercial products were analyzed with the simulated FIA method and enzymatic method. Oatrim is α-amylase hydrolyzed oat flour and Nutrim is jet-cooked oat bran (Inglett and Carriere, 2001). For enzymatic method, β-glucan Assay Kit by Megazyme was used. Experimental data showed good correlation between the two methods are shown in Fig. 8. These samples were further analyzed with SEC–FIA on-line post-column detection (Wood et al., 1991; Gomez et al., 1997; Knuckles et al., 1997) for the evaluation of MW distribution of β-glucan in the sample. Detailed description of this analysis will be presented in the subsequent report.

In this report, pH 10 buffer solutions were used throughout the experiments because Calcofluor does not easily dissolve in neutral water. And also, β-glucan dissolves in alkaline medium better than in neutral water. It was observed that the higher the pH, the higher solubility of glucan and Calcofluor. To avoid possible degradation of glucan at higher pH, however, pH 10 was chosen. According to Megazyme, β-glucan is stable even in 0.1 N NaOH solution. Since the intensity of fluorescence is dependent on the pH of the solvent medium, the use of buffer solution is indispensable.

4. Conclusions

Fluorescence measurements revealed that the fluorescence intensity of the Calcofluor/β-glucan complex was strongly dependent on the ionic strength of the solvent medium. For the Calcofluor-FIA method to be more reliable, it required that the low ionic strength solutions should be avoided. Higher ionic strength solution enhances the fluorescence of the low MW β-glucan to the level of the high MW β-glucan thereby extending the detectable low MW limit. The concentration of Calcofluor should be the same or higher than that of β-glucan in the FIA method to be viable.

From turbidity measurements, it was found that Calcofluor/β-glucan complex form aggregates. The formation of these aggregates was the reason why SEC has not been employed for the quantitative determination of Calcofluor/β-glucan complex. Concentration of Calcofluor and MW of β-glucans were the two major factors that govern the rate of aggregate formation. Higher MW β-glucans formed aggregates more rapidly than lower MW β-glucans. Although the aggregate formation itself does not normally interfere with the fluorescence intensity measurement, prompt measurement is recommended to avoid any errors caused by the precipitation of aggregates especially when the MW of β-glucan is very high.

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